Transmission of *Babesia caballi* by *Dermacentor nitens* (Acari: Ixodidae) Is Restricted to One Generation in the Absence of Alimentary Reinfection on a Susceptible Equine Host

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ABSTRACT The tropical horse tick, *Dermacentor nitens*, is a natural vector of *Babesia caballi* in the Americas. *B. caballi*, one of the etiologic agents of equine piroplasmosis, occurs widely throughout the world, but the United States and a few other countries are considered to be free of infection. *B. caballi* is transovarially transmitted by the one-host tick *D. nitens*; we tested the hypothesis that *B. caballi* can persist in multiple generations of *D. nitens* in the absence of opportunity to reacquire infection from a susceptible equine host. Partially engorged female *D. nitens* were collected from a *B. caballi*–infected horse in Puerto Rico and allowed to reattach and feed on an uninfected horse, successfully transmitting the infection. Three subsequent generations of ticks were reared on calves (nonsusceptible hosts for *B. caballi*), testing for *B. caballi* infection in each generation by feeding a sample of the larvae on naïve horses. The first generation of *D. nitens* reared on a nonsusceptible host transmitted *B. caballi*, whereas the second and third failed to transmit to naïve horses, showing that *D. nitens* infection with *B. caballi* was restricted to one generation in the absence of alimentary reinfection. These results imply that, in the event of the introduction of this pathogen into areas of the continental United States where *D. nitens* occurs, the tick could become a short-term reservoir of *B. caballi*, making control of introduced infections more complex.

KEY WORDS equine, tick-borne transmission, one-host tick, transovarial transmission, piroplasmosis

Babesia caballi (Nutall and Strickland 1910) and *Theileria equi* (Mehlhorn and Schein 1998) are tick-borne hemoprotozoan parasites that cause equine piroplasmosis. These pathogens have a worldwide distribution, but several countries are considered by the O.I.E. to be free of infection, among these are the United States, Australia, Japan, England, and Ireland (Brüning 1996, De Waal 1992). The economic impact of equine piroplasmosis results primarily from limitations on the international movement of horses. Import and export regulations often require serological testing of animals before movement because of the presumed presence of tick vectors in areas that are regarded as free of equine piroplasmosis (Brüning 1996, Schein 1988).

Throughout the world, several Ixodid tick species are competent vectors of *B. caballi;* in the Americas, the principal vector is *Dermacentor nitens* (Neumann). The distribution of *D. nitens* ranges from the southern parts of Florida and Texas in the United States to the north of Argentina. *D. nitens* is a one-host tick in which the adult females transmit *B. caballi* to their progeny transovarially (Roby and Anthony 1963, Roby et al. 1964), and all tick stages (larvae, nymph, and adult) are competent vectors (Stiller and Frerichs 1979).

There are many examples in the literature suggesting that certain species of *Babesia* are capable of persisting in their tick vectors for several filial generations even when fed on hosts that are not susceptible (Uilenberg 2006). Regarding *B. caballi*, the protozoan can persist for one generation in *Dermacentor silvarum* fed on cattle and sheep (Budnik 1941) and for seven generations in *Hyalomma plumbeum* (Abramov 1955).

The objective of this study was to test the capability of *D. nitens* to retain and transmit *B. caballi* in the absence of acquisition feeding on an infected host. To evaluate this, a laboratory colony of *D. nitens* was initiated with ticks collected from a *B. caballi*–infected horse in Puerto Rico. Three consecutive generations of *D. nitens* were reared on calves, a nonsusceptible

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host, and the larvae of each generation was tested for their ability to transmit *B. caballi* to naïve horses.

Materials and Methods

Animals. The mixed-breed horses used in this study were determined to be free of *B. caballi* by c-ELISA (VMRD, Pullman, WA) following the manufacturers' recommendations, and hemi-nested polymerase chain reaction (nPCR; described below). Because cattle are not known to be susceptible to *B. caballi* infection, Holstein calves were used for tick rearing. To confirm that they were not susceptible, calves were tested by nPCR for *B. caballi* before and after tick exposure. Animals were maintained at the USDA–ARS hemoparasite barn on the campus of the University of Idaho. All animals in this study were cared for following procedures approved by the University of Idaho Institutional Animal Care and Use Committee.

Experimental Design. Twenty-six partially engorged females and one male *D. nitens* were collected in Puerto Rico from a horse naturally infected with *B. caballi;* blood and serum samples were also collected. These ticks were allowed to reattach and feed to repletion on a naïve horse (Ho-84). Replete females from Ho-84 were held for oviposition at 26°C and 92% RH, with a 12 light:12 dark photoperiod. After oviposition was complete and the eggs had embryonated, all of the eggs laid by the 26 females were gently but thoroughly mixed with a spatula and divided into 1-g aliquots. After hatching, the larvae were maintained at 15°C, with the same relative humidity and photoperiod. Hatched larvae from this batch of eggs represented the parental (P_1) generation. Subsequently, the ticks were reared through three consecutive generations (F_1, F_2, F_3) on cattle, and the larvae from 2 g of eggs from each generation were fed on two horses (1 g/horse) to test for *B. caballi* infection (Fig. 1). Presence or absence of *B. caballi* infection in the horses was determined by c-ELISA (VMRD) and nPCR (described below).

Tick feedings were accomplished by placing all the larvae that hatched from 1 g of mixed eggs (≈20,000 larvae) under a cloth patch glued to the back of the host as previously described for *Boophilus* tick rearing (Ueti et al. 2005). The larvae were fed through to the adult stage. Test horses were monitored daily for clinical signs; blood (EDTA) and serum samples were collected and analyzed twice a week. Horses that were negative 75 d after tick exposure were challenged by intravenous subinoculation with fresh infected blood collected from the jugular vein (EDTA) of *B. caballi* chronic carriers to show susceptibility to *B. caballi.*

PCR. Genomic DNA was isolated from 100 μl of blood using the Puregene DNA purification system (Gentra Systems, Minneapolis, MN) and resuspended in 50 μl of TRIS EDTA buffer. Presence of *B. caballi* was detected by nPCR targeting the *rap-1* gene (Genbank accession number AF092736.1). The first-round PCR using external-forward primer 5′-GATTACTTGTCGGCTGTGTCT-3′ paired with reverse primer 5′-CGCAAGTTCTCAATGTCAG-3′ amplified a 375-base

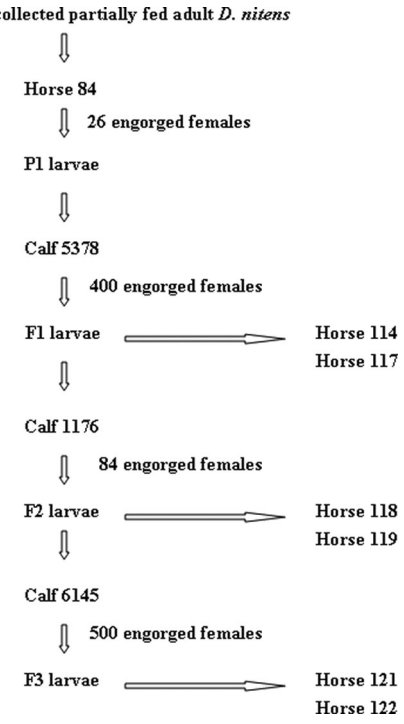

Fig. 1. Experimental design.

fragment from nucleotide 314–688. The second-round PCR used internal-forward primer 5′-GCTAAGTACCAACCGCTG A-3′ along with the same reverse primer to amplify a 222-base nested fragment from nucleotide 467–688. First-round reactions were performed in 25 μl with 5 μl of genomic DNA, 12.5 μl of PCR master (Roche Applied Science), and 2.5 μM of each external-forward and reverse primer. Second-round reactions contained 1 μl of the first-round reaction product, 12.5 μl of PCR master, and 7.5 μM of each internal-forward and reverse primer in 25 μl. The first round thermo-cycler program was 95°C for 5 min; 25 cycles of 95°C for 20 s, 60°C for 20 s, and 72°C for 20 s; final extension 72°C for 5 min; and holding at 4°C. The second-round program was 95°C for 5 min; 25 cycles of 95°C for 5 s, 60°C for 5 s, and 72°C for 5 s; final extension 72°C for 5 min; and holding at 4°C. To confirm the specificity of the nPCR assay, amplicons were sequenced in both directions using a Big Dye Kit and an ABI Prism automated sequencer as directed by the manufacturer (Applied Biosystems). To determine sensitivity, nPCR was carried out on DNA isolated from 10-fold serial dilutions of *B. caballi*–infected red blood cells from culture (Holman et al. 1993) (Fig. 2).

To confirm the presence of good-quality DNA, all samples that were negative for *B. caballi* by nPCR were tested with nPCR primers targeting horse β *actin* (GenBank accession number NM001081838). Using the same reaction mixture and thermo-cycler profile described previously for *rap*-1, horse β *actin* external forward 5′-TGCGTGACATCAAGGAGAAG-3′ and external reverse 5′-GTGTTGGCGTACAGGTCCTTA-3′

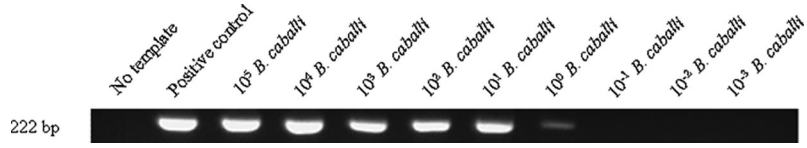

Fig. 2. Sensitivity of nested PCR targeting the *B. caballi rap*-1 gene. A 2% agarose gel stained with ethidium bromide showing nested PCR amplification of a 222-base fragment of the *rap*-1 gene from 10-fold serial dilutions of *B. caballi*–infected red blood cells from culture.

primers amplified a 264-base fragment; internal forward primer 5′-CCTCCTTCCTGGGCATGGAATC-3′ and the same reverse primer amplified a 100-base nested fragment. Presence of good-quality DNA was confirmed in all samples (results not shown).

Results and Discussion

The experimental horse (Ho-84) exposed to partially fed *D. nitens* collected from a *B. caballi*–infected horse in Puerto Rico showed clinical signs compatible with piroplasmosis (fever and depression) by days 7–10 after tick exposure, and infection was confirmed using nPCR and c-ELISA (Table 1). All 26 female ticks fed to repletion on Ho-84 and laid eggs. The resulting larvae were designated the parental or P_1 generation. P_1 ticks were subsequently reared through three consecutive generations (F_1, F_2, and F_3) on cattle (C-5378, C-1176, and C-6145, respectively). The number of engorged females collected from each generation is shown in Fig. 1. The first generation of ticks (F_1) reared on calf C-5378 transmitted *B. caballi* to two naive horses (Ho-114 and Ho-117); parasites were first detected in the blood at 20 and 14 d, respectively. Infections were confirmed by nPCR and c-ELISA (Table 1). In contrast, the second and third generation of ticks (F_2 and F_3), reared on calves C-1176 and C-6145, respectively, failed to transmit *B. caballi* to naïve horses. The F_2 generation was fed on Ho-118 and Ho-119 and the F_3 generation on Ho-121 and Ho-122. Horses fed on by F_2 and F_3 larvae were monitored for *B. caballi* infection using nPCR and c-ELISA and remained negative for 75 d after tick exposure (Table 1). These horses were challenged intravenously with infected blood from a *B. caballi* chronic carrier (10 ml for Ho-118 and Ho-119 and 100 ml for Ho-121 and Ho-122) to confirm that they were susceptible to *B. caballi* infection. Infection was confirmed by nPCR and c-ELISA in all four horses 7–13 d after the intravenous challenge (data not shown).

Testing serial dilutions of *B. caballi*–infected red blood cells showed that the technique was sensitive enough to detect DNA isolated from a single parasite (Fig. 2). Blood from the three calves (C-5378, C-1176, and C-6145) before and after tick exposure was negative for *B. caballi* by nPCR. Thus, we found no evidence of *B. caballi* infection of the calves used for feeding the P_1, F_1, and F_2 *D. nitens* tick generations.

In summary, our data showed that, after rearing *D. nitens* through three consecutive generations on nonsusceptible bovine hosts, they retained the ability to transmit *B. caballi* infection only during the first generation. Based on the transmission feeding of ≈20,000 larvae per horse, the F_2 and F_3 generations of ticks failed to transmit *B. caballi* to naïve horses, suggesting that this Puerto Rico isolate of *B. caballi* was only able to persist in *D. nitens* for a single generation in the absence of alimentary reinfection from an infected host. These results are consistent with previous published work showing that *B. caballi* remained in other ticks species such as *D. silvarum* (Budnik 1941) and *Hyalomma plumbeum* (Abramov 1955) for one or more generations.

One possible explanation for the retention of *B. caballi* infection in the F_1 generation of *D. nitens* could be co-feeding transmission between larval ticks. However, this would require that larvae become infected by sporozoites released from adjacent co-feeding larvae, and there are no reports in the literature suggesting that sporozoites are infective for other ticks, although this possibility should be studied further. Transmission of tick-borne pathogens between co-

Table 1. Presence of *B. caballi* in horses as determined by nPCR and c-ELISA before and after tick exposure

Horse	Tick generation	nPCR		c-ELISA	
		Before tick exposure	After tick exposure	Before tick exposure	After tick exposure
Ho-084	Field collected[a]	−	+	−	+
Ho-114	F_1	−	+	−	+
Ho-117	F_1	−	+	−	+
Ho-118	F_2	−	−	−	−
Ho-119	F_2	−	−	−	−
Ho-121	F_3	−	−	−	−
Ho-122	F_3	−	−	−	−

Ho-114 and Ho-117 tested nPCR positive 20 and 14 d after tick exposure, respectively. Ho-118, Ho-119, Ho121, and Ho122: remained negative until 75 d after tick exposure.

[a] *D. nitens* collected from the horse in Puerto Rico.

feeding infected and uninfected ticks in the absence of a systemic infection in the host has been described for several tick-borne viruses, as well as for *Borrelia burgdorferi* (Randolph et al. 1996). However, because of the biological differences between viruses, bacteria, and protozoa, we cannot assume that these phenomena might occur by the same mechanism. If co-feeding transmission was occurring, the F_2 and F_3 tick generations should have also become infected and transmitted *B. caballi* to the horses.

The persistence of *B. caballi* infection of *D. nitens* for a single generation has epidemiological implications in the event that the parasite is introduced into areas of the continental United States where *D. nitens* occurs. Taking the life cycle of the tick into account (Despins 1992, Díaz and De la Vega 2000), *B. caballi*–infected *D. nitens* could remain infected for >1 yr in the environment in the absence of susceptible equine hosts. In light of these results, it seems that *D. nitens* could become a short-term reservoir for *B. caballi*, making this hemoparasite more difficult to eradicate if it is introduced. Further studies need to be done to investigate the mechanisms that allow *B. caballi* to persist in *D. nitens* and other ticks species in the absence of alimentary reinfection on an infected host.

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